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Members of the myc oncoprotein family contribute to the pathogenesis of many human malignancies. Myc proteins are members of the bHLH-ZIP family of transcription factors that bind to DNA in heterodimeric association with another bHLH-ZIP protein, max. We originally proposed to develop an in vivo assay for myc-max association using green fluorescent protein (GFP)-tagged molecules. We suggested that formation of myc-max heterodimers might be associated with quantifiable fluorescence resonance energy transfer (FRET) and that loss of the FRET signal could be used as a way of screening for drugs that disrupt myc-max association. Although we have been unsuccessful in this regard, we have been quite successful in developing a highly sensitive yeast-based assay. Current efforts are focused on adapting this assay into a 96 well plate format that can rapidly and reproducibly be used for the screening of several low molecular weight compound libraries available to us. Over the next year, we hope to use this screening procedure to identify candidate drugs that will be further tested for their specificity and sensitivity in promoting myc-max dissociation.

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INTRODUCTION

Members of the Myc oncoprotein family are abnormally expressed in a variety of human malignancies, including Burkitt's and non-Burkitt's lymphomas, and breast, colon, and prostate cancer.[1] It has been estimated that over 70,000 cancer deaths per year in the United States are attributable to tumors which overexpress c-myc, the most widely studied member of this family.[2] In the case of prostate cancer, recent reports have suggested that 25-50% of all malignant prostate tumors may have elevated c-myc expression or activity.[3-5]

Myc oncoproteins are basic-helix-loop-helix-leucine zipper (bHLH-ZIP) transcription factors which bind to specific DNA sequences (E-boxes) in heterodimeric association with another bHLH-ZIP protein, max.[6] Many genes, including a number from our laboratory, have now been described which appear to be transcriptional targets for c-myc.[7-9] Most c-myc targets appear to encode proteins involved in cellular growth, metabolic, adhesive, and cell cycle properties. Together, these proteins begin to provide a framework within which to understand the means by which myc proteins are able to promote tumorigenic conversion.

Because of the large number of c-myc-regulated genes, inhibiting any one of them might not be an effective way to treat a myc-overexpressing tumor. Rather, a more proximal approach, aimed at disrupting actual c-myc-max heterodimers, might be a much more effective means of targeting their oncogenic potential as this could potentially inhibit all downstream targets. We therefore proposed in our original application to develop a system based on fluorescence resonance energy transfer (FRET) between c-myc and max proteins. Briefly, we proposed to fuse c-myc and max with different spectral variants of green fluorescent protein (GFP).[10] Upon co-expression of these proteins in mammalian cells, their heterodimerization could be indirectly quantified and monitored in real time by exciting one of the linked GFP variants and measuring emission at the wavelength of the other GFP spectral variant.[11] This would provide the basis for an assay in which a mammalian cell expressing GFP-c-myc and GFP-max could be used to screen for low molecular weight compounds capable of causing a reduction in FRET, and by extension, a disruption of c-myc-max interaction.

BODY

1. *Attempts to optimize the c-myc-max FRET signals in mammalian cells.*

The efficiency with which any two molecules is able to generate a detectable FRET signal upon dimerizing is highly dependent upon the distance between their respective fused GFP moieties.[11] Because c-myc is significantly larger than max (439 amino acids vs. 151 amino acids, respectively), it seemed unlikely that simply tagging each of the full-length molecules with GFP would generate any detectable FRET signal, let alone one of sufficient intensity to use in the development of our proposed model system (Fig. 1A). We therefore constructed a series of deletions, mostly of c-myc, designed to place GFP at varying distance from the bHLH-ZIP dimerization domain (Fig. 1B). The central idea was to optimize the intermolecular distance between GFP moieties in order to maximize the FRET signal. Several max variants were also designed, mostly to test the possibility that C-terminal regions of the molecule were inhibitory to the FRET signal. All of these mutants retained strong nuclear localization signals which are confined to the HLH domains of both c-myc and max.[12,13] Fluorescence microscopy of transiently transfected Cos-7 cells showed that all of the proteins localized to nuclei as expected (not shown). Despite robust expression of these proteins, in all possible c-myc-max combinations, we were unable to generate significant FRET signals.
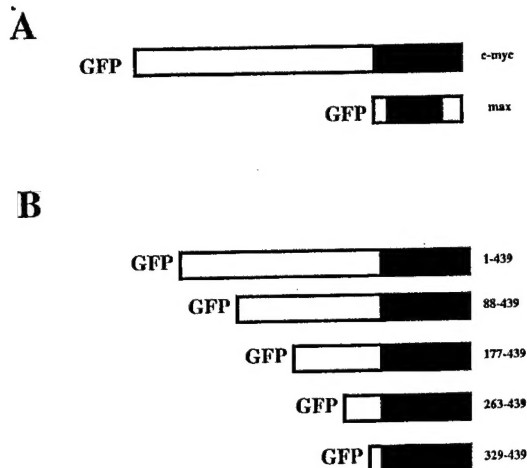
A

B

Fig. 1. A, Structure of c-myc and max proteins. The full-length, 439 amino acid c-myc and 160 amino acid max proteins are each depicted, with bHLH-ZIP dimerization domains indicated by filled regions. Each of these was expressed as full-length GFP chimeric proteins. In all cases, GFP was fused to the N-terminus of c-myc or max so as to minimize potential interference with c-myc-max heterodimerization; (B) Depiction of each of the GFP-c-myc mutants tested for FRET in association with max.

2. Development of a yeast-based 2-hybrid assay for c-myc-max interactions.

Our failure to develop an effective FRET-based mammalian cell assay for c-myc-max interactions has led us to pursue an alternate approach. Using a yeast 2-hybrid assay, we have previously shown that it is possible, with high sensitivity, to detect interactions between several other members of the bHLH or bHLH-ZIP family.[14-16] This assay is based on the *in vivo* expression in yeast of fusions between two mammalian proteins of interest with either the DNA binding domain or the transcriptional activation domain of the yeast Gal 4 protein. Interaction between the two mammalian proteins results in the creation of a bipartite transcription factor that is capable of both binding to and activating transcription of a stably integrated *lacZ* plasmid whose promoter contains a Gal4 binding site. We therefore adapted this technique to the detection of c-myc max interactions. As shown in Fig. 2, we were able to detect such an interaction when both c-myc and max proteins were co-expressed. No such interaction was seen when either protein was expressed individually. As we previously reported, a positive control, between the E12 and Id2 bHLH proteins also gave a strong interactions whereas neither of the individual proteins did so.[14] Because the c-myc protein that was expressed contained only the minimal bHLH-ZIP region, we conclude that the interaction between c-myc and max occurs via this domain in both proteins.
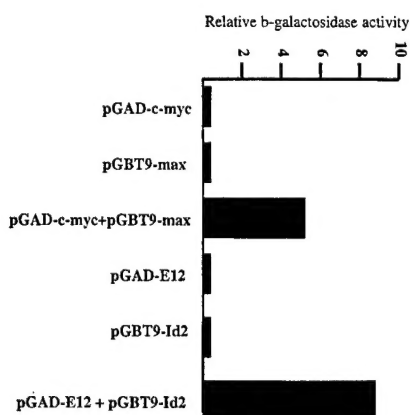

Fig. 2. β-galactosidase activity of yeast strains expressing the indicated plasmids. Yeast were grown in liquid culture, lysed, and assayed for β-galactosidase activity as previously described (14). The assay uses O-Nitrophenyl β-D-galactopyranoside (ONPG) as a colorimetric substrate

KEY RESEARCH ACCOMPLISHMENTS

- Establishment that FRET-based assays cannot be utilized to detect c-myc-interactions *in vivo*.

- Establishment that yeast-based 2-hybrid assays can be used to detect c-myc-max interactions *in vivo*.

REPORTABLE OUTCOMES

None at this time

CONCLUSIONS

Our original intentions were to develop a mammalian based assay that could be applied as a rapid means of screening for low molecular weight compounds that disrupt the *in vivo* interaction between c-myc and max. Although a number of different c-myc and max constructs were utilized, we have been unable to generate sufficiently strong FRET signals to permit the application of this approach for its intended purpose. Given that this assay was based upon transient transfections in Cos-7 cells in which the plasmids were allowed to replicate to high copy number, it seems unlikely that, even if the approach were to be eventually successful in this system, it would still be difficult to develop using stably transfected cell lines which express considerably lower levels of these proteins.

Although we have all but abandoned the mammalian-based approach, we have been quite successful in developing a yeast-based assay. Indeed, in retrospect, we see several distinct advantages of continuing to pursue this system:

1. yeast are easier and cheaper to maintain than mammalian cells;
2. because yeast grow much more rapidly than mammalian cells, it should be possible to perform assays in amore rapid manner;
3. because yeast contain no endogenous myc or max proteins, compounds which interfere with their interaction should be less likely to inhibit yeast cell growth. This could therefore expand the numbers of compounds which we ultimately identify as being potential "myc-max disrupters."

Over the next year, we intend to pursue several aspects of our yeast system with the intention of developing a robotically-based assay that will permit the high throughput screening of literally tens of thousands of low molecular compounds from several different commercial and proprietary libraries available through The Department of Pharmacology at The University of Pittsburgh Medical Center. This will be done is collaboration with Dr. Edwin Levitan, the Co-PI on this project.

1. We will establish conditions under which our "myc-max yeast" can be reproducibly grown to consistent saturation densities in microtiter plates.
2. We will attempt to enhance the sensitivity of the β-galactosidase assay by utilizing fluorescent rather then colorimetric substrates. Preliminary studies with one such substrate, fluorescein-Di-β-D-galactopyranoside (FDG-Molecular Probes, Inc, Eugene, OR) have provided encouraging results. Other fluorescent substrates are available and will be utilized as needed although, at the current time, we think it unlikely that it will be necessary to pursue these.

3. We will investigate a variety of approaches to the performance of β-galactosidase assays so as to minimize the number of manipulations and the amount of substrate (which is quite expensive).
4. With the above-described assay, we will establish conditions under which we can perform the proposed high throughput screens.

The tentative approach that we envision taking in 4.will be to inoculate microtiter plates with low-density cultures of yeast. The plates will next be inoculated with compounds from the library and incubated with shaking for a 1-2 day period. Automated plates readers will be used to distinguish those wells in which yeast have attained saturation density (i.e. no toxic effect of compounds) from those in which growth has been inhibited (compounds are toxic). β-galactosidase assays will be performed on the former set of wells according to the conditions established in 3. above and quantitated on a fluorescent plate reader. A "fluorescence to cell density ratio" will be obtained for each well. Those showing a significant reduction in this ratio will be tagged as containing compounds of potential interest. The above assays will then be repeated so as to establish the reproducibility of the findings and to establish optimal concentrations of the compounds being studied. β-galactosidase assays, utilizing such bHLH interactions as the E12-Id2 assay described in Fig. 2, will serve as controls for the c-myc-max specificity of any observed inhibitory compound.

REFERENCES

1. Nesbit CE, Tersak JM, Prochownik EV: Myc oncogenes and human neoplastic disease. Oncogene1999; 18:3004-3016.
2. Dang CV: C-Myc target genes involved in cell growth, apoptosis and metabolism (review). Mol. Cell Biol. 1999; 19: 1-11.
3. Jenkins RB, Qian J, Lieber MM, et al: Detection of c-Myc oncogene amplification and chromosomal anomalies in metastatic prostatic carcinoma by fluorescence in situ hybridization. Cancer Res. 1997; 57: 524-531.
4. Nuponnen NN, Kakkola L, Koivisto P, et al: Genetic alterations in hormone-refractory recurrent prostate carcinomas. Am. J. Pathol. 1998; 153: 141-148.
5. Prochownik EV, Grove LE, Deubler D, et al. Commonly occurring loss and mutation of the MXI-1 gene in prostate cancer. Genes, Chrom. & Cancer 1998; 22: 295-304.
6. Lüscher B, Larsson L-G: The basic region/helix-loop-helix/leucine zipper domain of Myc proto-oncoproteins: Function and regulation. Oncogene 1999; 18: 2955-2966.
7. Nesbit CE, Tersak JM, Grove LE, et al: Genetic dissection of c-Myc apoptotic pathways. Oncogene 2000; 19: 3200-3212.
8. Coller HA, Grandori C, Tamayo P, et al: Expression analysis with oligonucleotide microarrays reveals that Myc regulates genes involved in growth, cell cycle, signaling and adhesion. Proc. Natl. Acad. Sci. USA 2000; 97: 3260-3265.
9. Guo QM, Malek RL, Kim S, et al: Identification of c-Myc responsive genes using rat cDNA microarray. Cancer Res. 2000; 60: 5922-5928.
10. Tsien RY: The green fluorescent protein. Ann. Rev. Biochem. 1998; 67:509-544.
11. Dang CV, Lee WMF: Identification of the human c-Myc protein nuclear translocation signal. Mol. Cell Biol. 1988; 8: 4048-4054.
12. Kato GJ, Lee WMF, Chen L, et al. Max: functional domains and interactions with c-Myc. Genes Dev. 1992; 6: 81-92.
13. Langlands K, Anand G, Yin X-Y, et al. Differential interactions of Id proteins with basic-helix-loop-helix transcription factors. J. Biol. Chem. 1997; 272: 19785-19793.
14. Gupta K, Anand G, Yin X-Y, et al. Mmip-1, a novel leucine zipper protein that reverses the suppressive effects of Mad family members on c-Myc. Oncogene 1998; 16: 1149-1159.
15. Yin X-L, Gupta K, Han WP, et al. Mmip-2, a novel RING finger protein that interacts with mad members of the Myc oncoprotein network. Oncogene 1999; 18: 6621-6634.